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SUBSTRUCTURE OF THE CORTICAL SINGLET MICROTUBULES IN SPERMATOZOA OF *MACROSTOMUM* (PLATYHELMINTHES, TURBELLARIA) AS REVEALED BY NEGATIVE STAINING[1]

MARY BETH THOMAS[2] AND CATHERINE HENLEY

Department of Zoology, University of North Carolina, Chapel Hill, North Carolina 27514

The cortical singlet microtubules of the spermatozoa of certain flatworms have been circumstantially implicated in motility. The motile spermatozoa of *Plagiostomum* have cortical singlet microtubules and no axonemes (Christensen, 1961), and the bodies of the spermatozoa of *Dugesia* and *Bdelloura* (Silveira and Porter, 1965) and *Mesostoma* (Henley, Costello, Thomas and Newton, 1969) contain singlet microtubules and undulate independently of the two free flagella.

Following negative staining, the cortical singlets of the spermatozoa of some species of flatworms, namely the lungfluke *Haematoloechus* (Burton, 1966a, 1966b, 1970) and the polyclads *Stylochus* (Thomas, 1970) and *Notoplana* (Henley, in press), have a helical wall structure. There have been observed in these forms transitions to a protofibrillar configuration, which is the more typical configuration of the subunits in some negatively stained singlets (Gall, 1966), doublets (André and Thiéry, 1963; Grimstone and Klug, 1966; Pease, 1963) and triplets (Wolfe, 1970).

Because of the molecular dimorphism of the microtubules and their probable function in the motile process, cortical singlet microtubules are of interest as a model system for microtubule-associated motility. Therefore, knowledge of the size, number, and arrangement of the subunits making up the walls of the microtubules is important to understanding their function.

The number of subunits which occurs around the circumference of microtubules has been established for some species (for a review, see Arnott and Smith, 1969), but this is not the case for the cortical singlets of spermatozoa of the flatworms. From the results of rotational analysis, Burton (1966a) has suggested that there are 8 subunits around the circumference of the cortical singlet microtubules of the spermatozoa of *Haematoloechus*. Negative staining reveals the presence of 6 or 7 protofibrils for the cortical singlets of the spermatozoa of *Stylochus* (Thomas, 1970). However, the number of protofibrils seen in negatively stained preparations probably represents only a portion of the entire complex in most cases. This may be due to maceration of some of the microtubules (see Henley, 1970) or to superimposition of one half of the protofibrils on the other half (Burton, 1966b).

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[2] Present address: Department of Biology, Wake Forest University, Winston-Salem, North Carolina 27109.

The motile spermatozoon of *Macrostomum* contains cortical singlet microtubules and there are no free or incorporated axonemes (Fig. 1). Negative staining reveals that, typically, the subunits of the cortical singlets are helically arranged, and sometimes undergo a transition to the protofibrillar configuration; this latter configuration was found to terminate in at least 12 protofibrils for a number of singlet microtubules.

Materials and Methods

Specimens of *Macrostomum* sp. were collected from still water below the dam of University Lake near Chapel Hill, North Carolina. Whole animals were placed in a Columbia watchglass containing 1% aqueous phosphotungstic acid (PTA), pH 6.8. The animals were teased with steel needles, and drops of the PTA, containing spermatozoa freed from the animal, were transferred to Formvar-

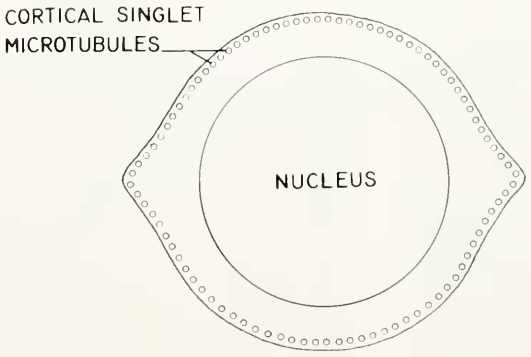
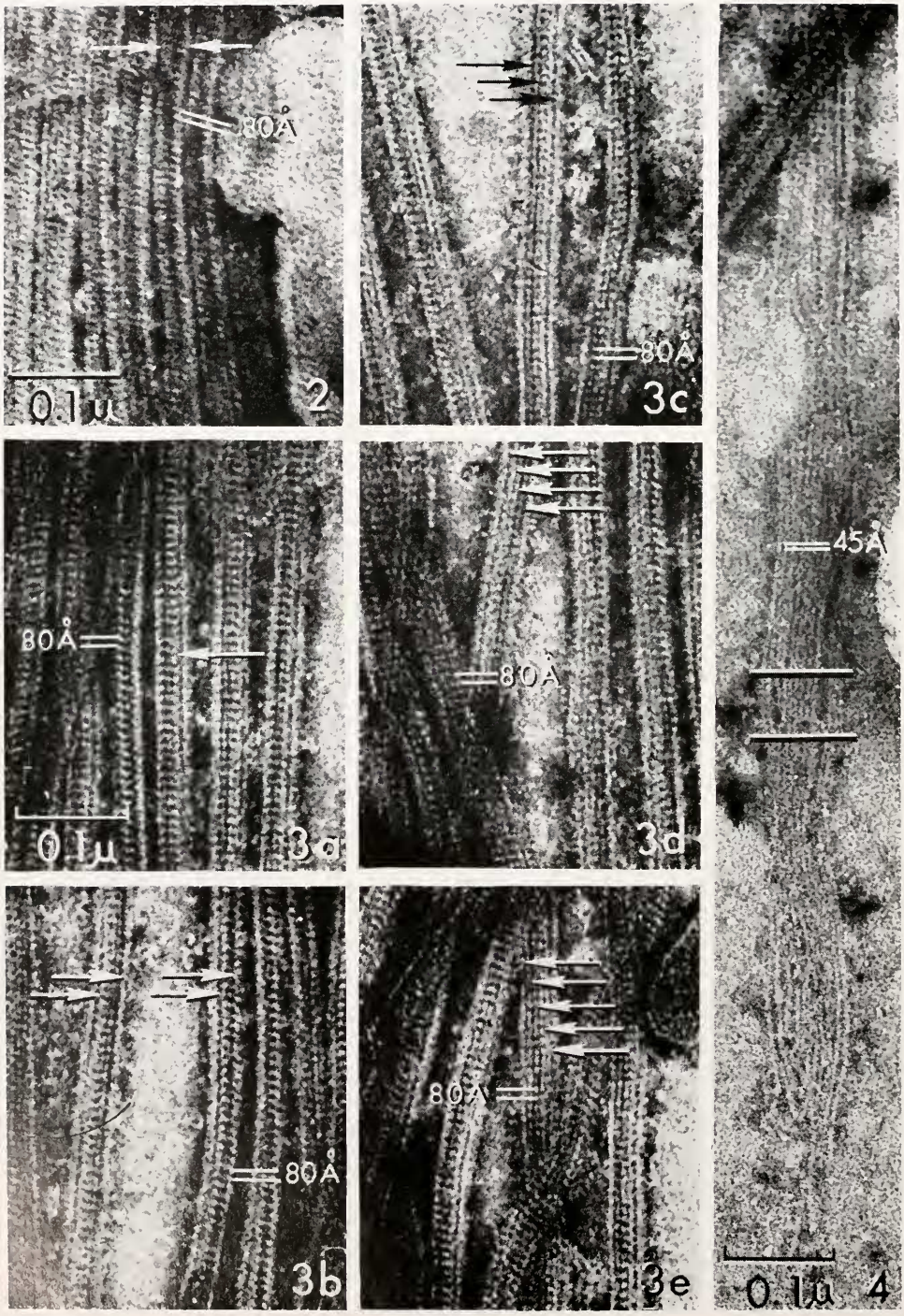


Figure 1. Diagram of the arrangement of cortical singlet microtubules in the nuclear region of a spermatozoon of *Macrostomum*. No axonemes are present.

carbon-coated 200-mesh copper grids. After approximately 6 minutes, the fluid was drained from the grids which were then allowed to dry; preparations were examined with the Zeiss 9A electron microscope.

Results

In sectioned spermatozoa of *Macrostomum*, there are *ca.* 60–80 cortical singlet microtubules; 30–40 were present in material negatively stained with PTA (the remainder presumably having been digested away or lost spatially from the total complement during the process of negative staining). Three arrangements of the subunits in these PTA-treated microtubules were found. (1) In a few regions, the subunits were in the intact helical configuration (Fig. 2), comparable to that described for cortical singlet microtubules in spermatozoa of other flatworms. (2) There were large areas in which the lateral spacing of the subunits was increased, but the circumferential integrity of the tubules appeared to be preserved in such a way that only the top halves of the microtubules could be seen (Fig. 3a-e). (3) At their termini, these microtubules ended in protofibrils lying in one plane (Fig. 4).



FIGURES 2–4.

(1) The width of the microtubules having the subunits arranged in the most compact helical configuration (Fig. 2) is *ca.* 200–210 Å; the alternating electron-lucent and electron-dense bands have a center-to-center spacing of *ca.* 80–85 Å and are inclined at an apparent angle of *ca.* 12–14°. The subunits appear to be closely apposed along the helical path, for there is no line of demarcation between adjacent subunits. Accumulation of the electron-dense PTA along the central axis suggests the presence of a lumen, indicating that the microtubule is cylindrical or only slightly flattened. Measurement of the cortical singlets in sectioned material likewise indicates a diameter of *ca.* 200 Å.

(2) Varying degrees of lateral separation of adjacent subunits occur in other regions. In those showing the least change from the helical configuration (Fig. 3a), one protofibrillar element is separated laterally from the remainder of the microtubule, which retains its helical arrangement of subunits. The overall width of the microtubule here is *ca.* 260 Å and there is a *ca.* 50 Å gap between the separated protofibril and the remainder of the microtubule; the helical portion is approximately 180 Å wide.

The opposite extreme of separation of the subunits is shown in Figure 3e, in which there are 6 protofibrils with a lateral center-to-center spacing of 50–60 Å. The overall width of the microtubule has increased here to 370 Å.

Intermediate stages between the two extremes are numerous and four examples are shown in Figures 3b-e, where arrows indicate two, three, four and five lateral separations, respectively. Analysis of the intermediate stages indicates that whereas the lateral spacing between protofibrils varies from 50 to 70 Å, the center-to-center spacing between subunits along the length of the protofibrils is much more constant, with the 80 Å spacing characteristic of the helical configuration being maintained, as noted on the figures.

(3) The third arrangement of subunits is in the protofibrillar configuration (Fig. 4). This differs significantly from the protofibrillar arrangement described above (2) following lateral separation of the protofibrils, and also from the protofibrillar configuration described for cortical singlets in spermatozoa of other flatworms. The maximum number of protofibrils previously reported for negatively stained cortical singlets is 6 or 7 (Thomas, 1970), but in Figure 4, a complement of 12 protofibrils can be seen for a single microtubule. The overall width of the ribbon of protofibrils here is *ca.* 650 Å and the minimum lateral separation of the protofibrils is 45–50 Å. Furthermore, in contrast to the 80 Å longitudinal center-to-center spacing of subunits along the separated portions of the microtubules as described in (2) above, the longitudinal spacing here is 40–45 Å.

FIGURE 2. Intact cortical singlet microtubules (arrows) of a spermatozoon of *Macrostomum*, showing the compact helical configuration of subunits typical of the cortical singlets of some of the platyhelminths; magnification: 152,000 ×.

FIGURE 3. Cortical singlet microtubules of a spermatozoon of *Macrostomum*, showing examples of progressive stages in the lateral separation of the protofibrils. Figures 3a, b, c, d, and e show one, two, three, four and five lateral separations, respectively. In each figure arrows indicate the region of the microtubule representative of the stage. Other patterns can often be seen in adjacent regions of the same microtubule and in adjacent microtubules; magnification: 152,000 ×.

FIGURE 4. Cortical singlet microtubules of a spermatozoon of *Macrostomum* in which the subunits are in the protofibrillar configuration. Twelve protofibrils can be counted in the region between the two lines; magnification: 152,000 ×.

FIGURE 5a. Terminal portions of 17 cortical singlet microtubules. The appearance of two helical structures for each microtubule could result if the top half of each microtubule slid off the bottom half while the microtubule was still in the helical configuration. The arrow indicates the region in which the width is *ca.* 460 Å. A single protofibril is associated with the right half-microtubule in each case; magnification: 62,700 ×.

Figures 5a and 5b show regions in which the top halves of the microtubules have slid off the bottom halves, the subunits remaining in the helical configuration. Superficially observed, the microtubules here appear to branch into two (Fig. 5a, arrow) microtubules; however, there is no accumulation of the negative stain along the central longitudinal axis of each "subtubule," and therefore no indication of the presence of a lumen. The width of the "double" structure at the point indicated by the arrow is *ca.* 460 Å. The width following 5 lateral separations (Fig. 3e) was *ca.* 370 Å, and for one lateral separation the overall width was usually *ca.* 260 Å. In Figure 5a and adjacent micrographs (not shown) of the entire complement of microtubules, each of the 17 "paired" structures can be traced to 17 intact singlet microtubules. Of interest is the observation that in all 17 microtubules shown in Figure 5a, a single protofibril, of slightly less electron density than the remainder of the structure, is invariably located on the same side of all 17 microtubules (Fig. 5b, arrows). The significance of this observation is not known.

Discussion

It has been suggested that the cortical singlet microtubules of the spermatozoa of *Plagiostomum* (Christensen, 1961), *Dugesia* and *Bdelloura* (Silveira and Porter, 1965) and *Mesotoma* (Henley *et al.*, 1969) are involved in motility. The cortical singlets from spermatozoa of *Haematolocchus* (Burton, 1966a, 1966b), *Stylochus* (Thomas, 1970) and *Notoplana* (Henley, in press) have been shown to have a helical arrangement of subunits. *Macrostomum* is the only species described thus far in which it is known that the spermatozoa are motile, with cortical singlets as their only microtubular component and with the subunits of the singlets helically arranged.

The transitions thus far described in the literature, from the helical to the protofibrillar configuration in cortical singlet microtubules, are usually quite abrupt (Burton, 1966a, 1966b; Thomas, 1970). In the cortical singlets described here, however, many stages in the transition are evident, providing a unique opportunity for understanding the event. This variability is undoubtedly the consequence, at least in part, of the macerating action of PTA (Henley, 1970), which makes feasible a study of the transitions in their various manifestations. Analysis of the changes which occur in the transition from the helical to the protofibrillar configuration suggests that two separate events occur. One change involves a lateral separation of the subunits to produce *ca.* 12 protofibrils, with a variable lateral spacing of 50–70 Å and a longitudinal center-to-center spacing of *ca.* 80 Å of subunits along the protofibril. The separation of any two adjacent protofibrils appears to occur at random, as evidenced by the wide variety of patterns shown in Figures 3a–e, and diagrammed in Figure 6. The diagram is based on the possible occurrence of 12 protofibrils, with the top six visible. Similar variability can, of course, be obtained with other numbers, but the observed range of patterns can be interpreted only with 11, 12 or 13 protofibrils.

The second change is in the spacing of the subunits along the length of a protofibril. In the helical configuration the subunits are spaced *ca.* 80 Å center-to-

Figure 5b. Enlargement of the area outlined by the rectangle in Figure 5a, showing the two helical portions and associated single protofibrils (arrows); magnification: 152,000 ×.

center along the length of the microtubule. This 80 Å spacing is maintained during lateral separation. However, in a truly protofibrillar configuration, such as that shown in Figure 4, the center-to-center spacing of the subunits is 40–45 Å. The possibility that this is a visual effect, produced by superimposition of protofibrils, is negated by the fact that the protofibrils are lying in parallel. Although

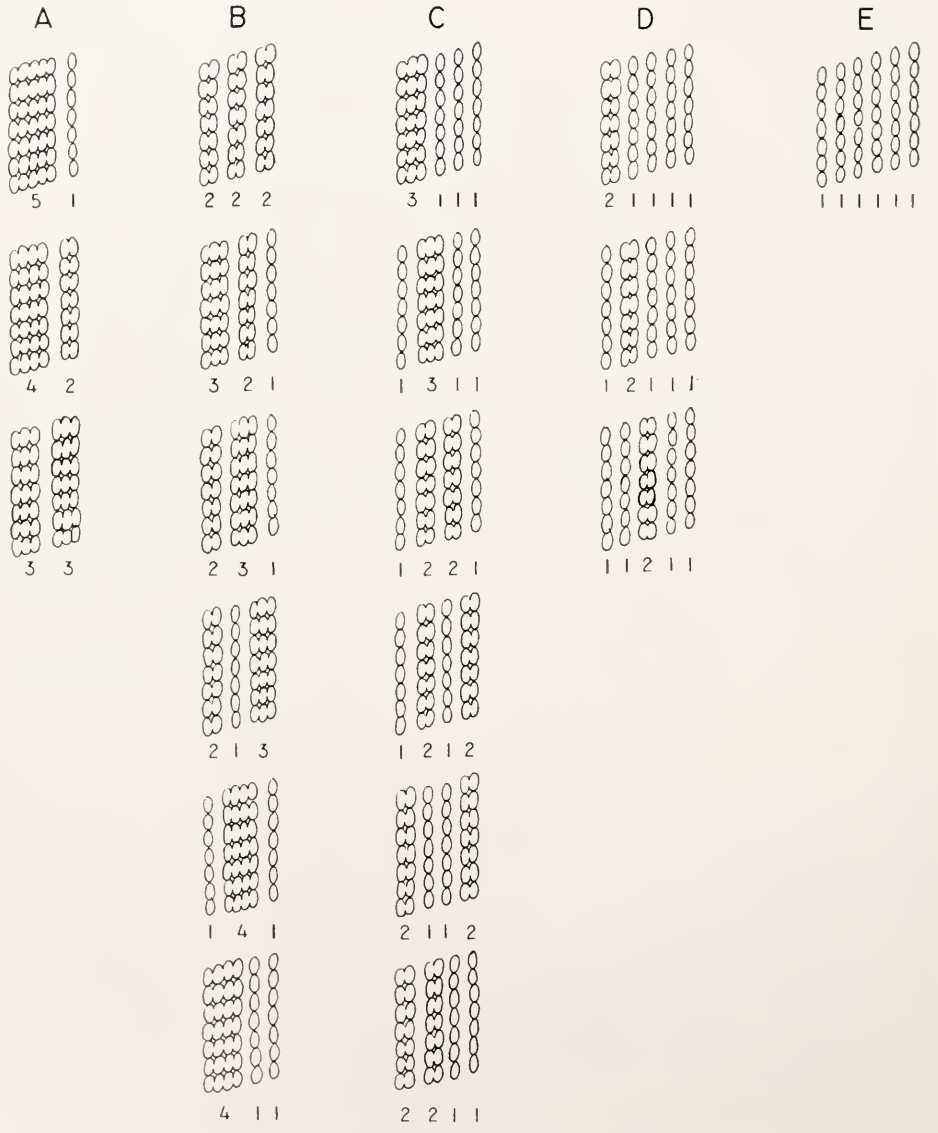


FIGURE 6. Diagram illustrating the possible array of patterns that could be exhibited by a microtubule if there are 12 protofibrils, of which the top 6 are visible, and if lateral separation of protofibrils occurs at random. Mirror images are not illustrated.

lateral separation can occur without a concomitant change in the longitudinal spacing, the change from 80 Å to 40–45 Å has never been observed by us to occur in the absence of lateral separation.

The shape and associations of the repeating units along the length of the intact microtubules in the helical configuration are not known. There are at least three

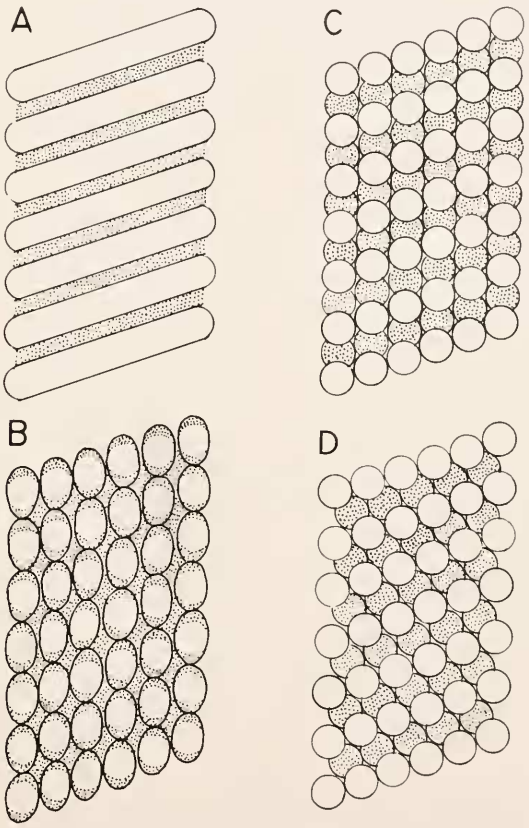


FIGURE 7. (A) Diagram of an intact cortical singlet microtubule as it appears following negative staining with PTA. The center-to-center spacing between adjacent white bands in this, as in (B), (C) and (D), is 80 Å; (B) Helically arranged ovoid subunits, joined end to end along the length of the protofibrils, as they might appear following negative staining; (C) and (D) Helically arranged globular subunits with alternating subunits slightly displaced toward the lumen of the microtubule, either parallel to the long axis of the microtubule (C) or nested among the outer subunits (D).

possible models that could explain the presence of the alternating electron-lucent and electron-opaque bands which repeat at intervals of *ca.* 80 Å along the length of the microtubule (Fig. 7A). One such alternative is based on the assumption that the walls of the microtubules are made up of ovoid subunits which are *ca.* 80 Å in length and joined end to end along the length of the protofibrils (Fig. 7B). In the region in which contact between adjacent subunits occurs, a slight

depression or groove would be formed in which the electron-opaque negative stain would accumulate, thereby producing alternating light and dark bands with an 80 Å repeat. The observed shift in spacing between subunits along the long axis of the microtubule, from the 80 Å periodicity in the intact microtubule (Fig. 2) to the 40–45 Å spacing in the truly protofibrillar configuration (Fig. 4), is in keeping with such a model of longitudinally oriented, elongated subunits, if a dimer-monomer relation were involved as well as a conformational change.

Two other models take into account the shift in spacing between subunits along the long axis of the microtubule. In one such model, alternate subunits along a protofibril are recessed slightly (about 10% of their diameters) toward the lumen of the microtubule (Fig. 7C). The recessed subunits could lie parallel to the protofibril (Fig. 7C) or nested among the outer subunits (Fig. 7D). In either case there would be formed around the microtubule a groove which would accumulate the electron-dense negative stain and produce the alternating bands with a repeat of 80 Å. A more widely spaced arrangement of outer subunits than those portrayed in Figures 7C and 7D is required here, since the recessed subunits would make a cylinder of smaller diameter.

There is biochemical evidence that the shift in spacing of subunits, from 80 Å in the intact microtubule to 40–45 Å in the protofibrillar configuration, may involve dissociation of dimers to form monomers along the length of the protofibrils. Shelanski and Taylor (1968) have reported that the subunits of microtubules in sea urchin sperm flagella can be isolated as dimers with a molecular weight of *ca.* 120,000 (and a corresponding particle size of 40–50 × 80–90 Å), or as monomers with a molecular weight of *ca.* 60,000 (and a particle size of 40 Å). If their biochemical results reflect the existence of dimers and monomers in the intact microtubule, then it is of interest to relate the occurrence of such configurations to the results observed here. The random nature of the lateral separation of subunits described gives no indication that monomers associate into dimers along the helical path (at an oblique angle to the long axis of the microtubule). However, the described shift in spacing of the subunits along the length of the protofibrils may be suggestive of an association of monomers to form dimers along the length of the protofibrils. Within such a framework, the formation of the groove to produce the alternating bands in the intact microtubule might be explained. If two monomers form an ovoid dimer with the long axis parallel to the long axis of the microtubule, the appearance might be similar to that shown in Figure 7B. Similarly, if one half of the dimer is inclined toward the lumen of the microtubule, the groove might be formed in a manner similar to that shown in Figures 7C and 7D. It is possible that freeze-etch or shadow-casting may give some information as to the contours of the subunits. Such experiments are in progress and may be useful in deciding among the possible alternatives.

Summary

1. Cortical singlet microtubules are the only microtubular components of the motile spermatozoa of *Macrostomum*. When negatively stained with phosphotungstic acid, the microtubules display a helical arrangement of the subunits similar to that described for spermatozoa of other species of platyhelminths.

2. The protofibrillar configuration of subunits of the cortical singlets can also occur under conditions of negative staining. One microtubule was clearly observed to be made up of 12 protofibrils.

3. Analysis of various stages in the transition from the helical to the protofibrillar configuration suggests that there are two steps in the conversion. These are (a) a seemingly random lateral separation of subunits to form protofibrils with a longitudinal periodicity of *ca.* 80 Å, which is characteristic of the periodicity of the intact helical arrangement, and (b) a subsequent change in spacing of the subunits along the length of the protofibril, from *ca.* 80 Å to 40–45 Å. These observations support the view that if monomers associate to form dimers, the dimers occur along the length of the protofibril rather than between adjacent protofibrils.

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